ISOELECTRIC FOCUSING AND FRACTIONATION AMPHOLYTES IN THERMALLY ENGENDERED PH GRADIENTS

BACKGROUND OF THE INVENTION

Fluids which are of interest to the physician, biologist, biochemist and chemist, as well as to the microbiologist, are often complicated mixtures containing some components in solution and others in suspension. The separation and characterization of these components constitute a problem of basic importance. Physical techniques such as ultra-centrifugation and electrophoresis, are most widely used prior art general methods for accomplishing the aforesaid separation and characterization. The process of the present invention constitutes an improvement over the prior art methods, whereby the components of a given mixture are separated from each other simultaneously and are sorted into a spatial arrangement referred to as an "isoelectric spectrum." The term "spectrum" is used herein to designate a sorting in the same sense as one refers to "mass spectra," "energy spectra," and the like. In the separation of ampholytes, such as proteins, polypeptides, amino acids, and the like, special use can be made of the existence of an isoelectric point, in order to obtain particularly sharp and stable "spectra."

The principle of the prior art process is based on the fact that ions of proteins, as well as other ampholytes, are positively charged at pH values below the isoelectric point and negatively charged at the higher pH values. By creating and maintaining a field of varying hydrogen ion concentration in a solution traversed by an electric current, it is possible to make the ampholytes converge from both sides towards points at which the pH value is equal to the isoelectric pH of the ampholyte. Such concentration of the ampholyte at its isoelectric point occurs when the current flows in the direction of increasing pH in the pH field, whereas a diminution in the ampholyte concentration at a point corresponding to its isoelectric pH is produced by a current flowing in the direction of diminishing pH values.

For every ampholyte there is a characteristic pH with which the net charge of the molecules is zero, this being referred to as the "isoelectric point." As the protein molecules proceed from the isoelectric point towards increasing pH values, they acquire a negative charge which increases with increasing pH. On the other hand, when the protein molecules proceed towards the acid side of the pH gradient, that is, toward decreasing pH values, they acquire a positive charge which increases as the pH value diminishes.

An electric current flowing through the electrolytic solution in the direction of increasing pH values will cause the positively charged ampholyte ions which are on the acid side of the isoelectric point to move with the current toward the isoelectric point. The ampholyte ions on the basic side of the isoelectric point are negative charged, and hence move contrary to the direction of the current, that is, they also move toward the isoelectric point. Thus the ampholyte ions converge towards the isoelectric point, where all the ampholyte particles can be eventually concentrated. Since different ampholytes have, as a rule, different isoelectric points, the various components of an ampholyte mixture may be separated by concentrating them at their characteristic isoelectric point. The sorting pattern thereby obtained may be referred to as an "isoelectric spectrum."

Electrophoretic mobility has been used for decades as a basis for the separation and characterization of proteins. The resolving power of separations is not high and the characterization by mobility is not unique because of the dependence of the protein mobilities on the pH and ionic strength of the buffers.

In **1954** a new method was proposed for the separation and characterization of proteins on the basis of differences in their isoelectric point (A. Kolin, Journal Chem. Phys., **22** 1268 (1954); **41** 101 (1955). This approach led in subsequent modifications to unique characterization of proteins and to high resolution in preparative separations (Svensson, *Acta Chem. Scand.* **15**, 325 (1961); **20**, 820 (1966); Kolin, pH Gradient Electrophoresis "Methods in Medical Research" Volume 12 1970)).

Proteins differeing by as little as 0.01 pH unit in their pI values could be separated by the aforesaid prior art methods. The protein is distributed, for example, in an electrophoretic column in which a pH gradient is maintained by a concomitant density gradient used for stabilization against thermal convection. An electric current directed toward increasing pH sweeps the protein which is positively charged in parts of the column where the pH is less than the pI toward the isoelectric zone where it is arrested due to the loss of charge. Similarly, protein ions which are negatively charged in the regions where the pH is greater than the pI are swept in the direction opposite to the current toward the isoelectric zone where they come to a stop. Thus, eventually all of the protein is condensed in a sharp isoelectric zone whose pH can be measured to ascertain the isoelectric point of the protein. A plurality of protein components differing in their pI value result in a corresponding plurality of sharp isoelectric condensation zones.

In an initial approach, as described in the aforesaid J. Chem. Phys. Proceedings the pH gradient was prepared by appropriate buffer mixture. Rapid isoelectric condensations, or "focusing," have been obtained in such prior art processes in less than 5 minutes. The weakness of this particular prior art implementation of the isoelectric focusing concept is the drift of the pH gradient which makes it impossible to reach a stable, steady-state pattern with each isoelectric protein zone created precisely at its isoelectric point within the pH gradient.

The aforesaid instability was removed in the subsequent prior art processes by creating a stable steady-state pH distribution through achievement of an equilibrium between electromigration and diffusion of buffer ions. This subsequent prior art process, however, is time consuming, in some cases requiring as many as 96 hours. In addition, this particular prior art process requires costly synthetic ampholytes, known as "Ampholines" to buffer the fractionation column. The Ampholines must be removed from the collected fractions by dialysis at the end of the process, and their absorption of ultraviolet light may interfere with the spectro-photometric evaluation of the fractionation.

The process of the present invention constitutes an improvement over the prior art by achieving isoelectric focusing and fractionation stability within a short time span and with a high and easily adjustable resolving power, and without the requirement for the special buffering material such as the "Ampholines."An initial implementation of the process of the invention includes a simple U-tube apparatus filled with a common buffer, such as tris, adjusted to a pH of intermediate value within a range between the highest and lowest pI of the components to be fractionated. A pH gradient is then generated by purely physical means within a few seconds so that isoelectric focusing can begin practically immediately leading to separations within several minutes and within a pH range of about 1 pH unit. The essence of the present invention is the generation of this pH gradient by the establishment of a temperature gradient in the electrophoretic column.

The process of the invention offers a simple and rapid means for identifying and separating different ampholytes such as proteins, polypeptides, amino acids, and the like, in complex mixtures. A method of condensation, or focusing, of extended volumes of mixtures of proteins, or other ampholytes, into an isoelectric spectrum is provided by the process of the invention; the spectrum comprising sharp narrow zones located at points of a pH gradient corresponding to the isoelectric pH values of the individual proteins.

As mentioned briefly above, the process of the invention is based on the establishment of a column of electrolytic solution in which a pH gradient has been established, and which contains an ampholyte in solution, or a suspension of ampholyte-coated particles. The charge on the protein ions, being a function of the pH of the solution, will vary throughout the pH gradient